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# EXPLORING THE BIOLOGICAL PROTECTIVE ROLE OF CAROTENOIDS BY RAMAN SPECTROSCOPY: MECHANICAL STRESS OF CELLS

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**ABSTRACT.** Carotenoids present a group of tetraterpenoid biomolecules which are well known for their physiological benefits through their remarkable radical scavenging activity and reactive oxygen species quenching. However, little is known about role of carotenoids in relieving mechanical stress. Hence, in this study, we exposed sea urchin fertilized eggs to mechanical stress in form of centrifugation and exploited the advantage of Resonance Raman scattering to probe if the carotenoid profile or their distribution would change after application of the stress. Silver nanoparticles were used ro probe the Raman signal of carotenoids near the cell surface and attempt to achieve SERRS conditions (Surface-enhanced Resonance Raman scattering). We have found that carotenoid concentration notably increased on the cell surface after centrifugation, indicating that carotenoids were mobilized in response to mechanical stress.

***Keywords:*** *Raman spectroscopy, mechanical stress, sea urchin eggs, carotenoids*

## INTRODUCTION

Silver nanoparticles (AgNPs) are often used in micro-analytical Raman spectroscopy for detection of SERS (Surface-enhanced Raman scattering) signal from cells and tissues owing to their relatively strong electromagnetic field enhancement

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by surface plasmon resonance (SPR), [1-6]. It has been shown previously that distinct Raman and SERS signal of carotenoids can be recorded from live cells and tissue extracts by AgNPs [2-6]. Although chemical protective role of carotenoids is well studied and discussed [7,8], the potential involvement of carotenoids in responses to other kinds of stress, like mechanical insult, is not well investigated yet.

Sea urchins are marine grazing herbivorous invertebrates (Fig. 1). Adult sea urchins are generally resistant to organic and heavy metal pollution [9]. However, the majority of sea urchin species are broadcast spawners, meaning that both the males and the females release their gametes (reproductive cells) freely into sea water [9], where the fertilization and early development takes place. Hence, their early life stages, the gametes, embryos and larvae are directly exposed to environmental chemical and physical factors.

**Fig. 1** Photograph of sea urchins in their native environment.

Our previous study has shown that Raman spectroscopy probing of native, mature eggs of the sea urchin *Paracentrotus lividus* induces pre-resonant excitation of carotenoids, whereby the Raman signal represents a mixture of mainly echinenone

and β-carotene [8]. The next developmental step after egg maturation is the fertilization, whereby a rigid protective envelope, composed of proteins and mucopolysaccharides, is raised around the eggs [10,11]. The purpose of the fertilization envelope is, *inter alia*, to protect the developing embryo from the physical environment. In this study, we investigate local changes of carotenoid profile or distribution in the sea urchin *Paracentrotus lividus* eggs after application of mechanical stress, in order to study the involvement of carotenoids in response to this kind of aggression.

## EXPERIMENTAL

### *Obtaining of sea urchin eggs*

Sea urchin gametes were obtained by artificial spawning of a male and of three females by injection of KCl solution (1 M). Male seminal fluid was collected "dry", while females were inverted on a beaker to release eggs into filtered natural sea water (filtering with Milipore vacuum filtration system, 0.45 μm pore size). Gametes were subsequently combined and gently stirred to promote fertilization. 5 minutes later, fertilized eggs were sieved through a 50 μm mesh to remove remaining gametes and debris. Optical micrographs of eggs were taken on an Olympus IX71 inverted microscope. More details on the employed spawning method can be found in references 6,8 and 12. Sea urchins were returned to the sea at the site of collection once the gametes have been collected.

### *Exposing the eggs to mechanical stress*

Obtained clean suspension of fertilized eggs was subjected to mechanical stress by centrifuging for 10 minutes at 2000 rpm in a lab centrifuge (50 ml tube capacity), firstly to obtain a concentrated mass of intact eggs, and further to evaluate the possible physiological reaction of centrifuged eggs relative to non-centrifuged ones. In order to localize Raman signal probing to cell surface and eliminate strong carotenoid signalling from egg interior, concentrated egg suspension was drop-coated onto SpectRIM hydrophobic plate, and covered by a droplet of colloidal AgNPs after water evaporation.

### *Synthesis of AgNPs*

The hydroxylamine-reduced colloidal AgNPs were prepared by dissolving of 0.017 g of $AgNO_3$ in 90 ml of distilled water [13]. A second solution was prepared by dissolving of 0.021 g of $NH_2OH \cdot HCl$ in 5 ml of water, and 4.5 ml of 0.1 M sodium hydroxide was further added. The second solution was rapidly added to first, $AgNO_3$ solution, and a grey-brown solution with absorption maximum at 418 nm was obtained in a few seconds.

### *Raman spectroscopy measurements*

Raman spectroscopy measurements of dry sea urchin eggs deposited on a SpectRIM plate by drop-coating method [8] was conducted with a Renishaw InVia Reflex confocal Raman microscope, using the Cobolt DPSS laser emitting at 532 nm. The 20x objective (NA 0.35) was used for viewing the sample and focusing the laser beam. This configuration resulted in the laser spot size of about 1 µm. Spectra were recorded with 0.5 $cm^{-1}$ spectral resolution, in ~100 to 1800 $cm^{-1}$ range. In both Resonance Raman and SERRS measurements, spectra were acquired by a single 1 s exposure under 100 mW laser power.

This study did not require ethical approval. However, all applicable international, national, and/or institutional guidelines for the care and use of experimental animals were followed.

## RESULTS AND DISCUSSION

The fertilized eggs were spherical in shape, with the diameter of about 90 µm, and yellowish to orange in colour owing to high content of carotenoids [8] (Fig. 2). The fertilization envelope was observed as a thin layer around the eggs (Fig. 2c). It forms a physical coat around the eggs with about 10 µm of perivitelline space between the envelope and egg cell membrane.

The normal Raman signal of centrifuged fertilized eggs featured only strong pre-resonance carotenoid signal on top of characteristic weak incremental background (Fig. 2a, red spectra), a signal described previously by Nekvapil et al. [8]. This means that the fertilization envelope enables the passage of incident laser beam and the scattered photons. The three main bands were observed here at 1004, 1154 and 1514 $cm^{-1}$, which in this case indicates a carotenoid mixture of echinenone and β-carotene [8].

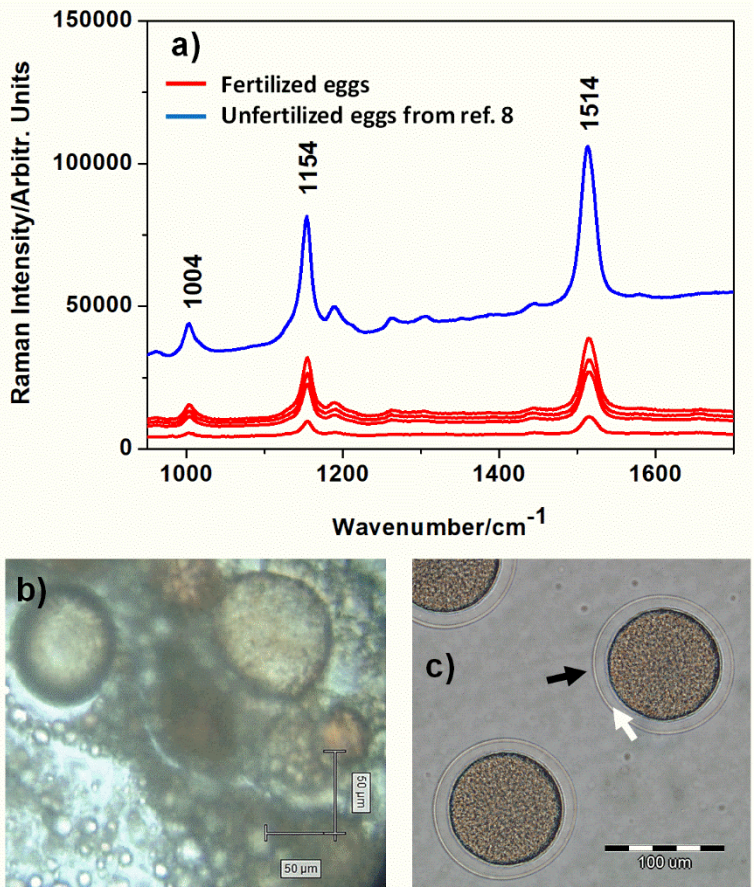


**Fig. 2** Analysis of native sea urchin *Paracentrotus lividus* eggs: a) multiple pre-resonance Raman spectra acquired from native fertilized eggs (red), compared to averaged Raman signal of unfertilized eggs from ref. 8 (blue); b) micrograph of unfertilized eggs taken on the Raman microscope; c) micrograph of fertilized eggs taken on inverted brightfield microscope (black arrow - fertilization envelope, white arrow - perivitelline space).

On the contrary, SERRS signal of uncentrifuged fertilized eggs (Fig. 3) were notably complex, presumably reflecting the numerous modes of the envelope material adsorbed onto AgNPs. The modes around 141 and 220 $cm^{-1}$ indicate the aggregation of AgNPs [4]. In between the multitude of bands, SERRS modes at 1079, 1174, 1367, 1510, 1575 and a shoulder at 1649 $cm^{-1}$ could be distinguished, highlighted by red labels in Fig. 3, which are close to SERS modes of β-carotene [5].

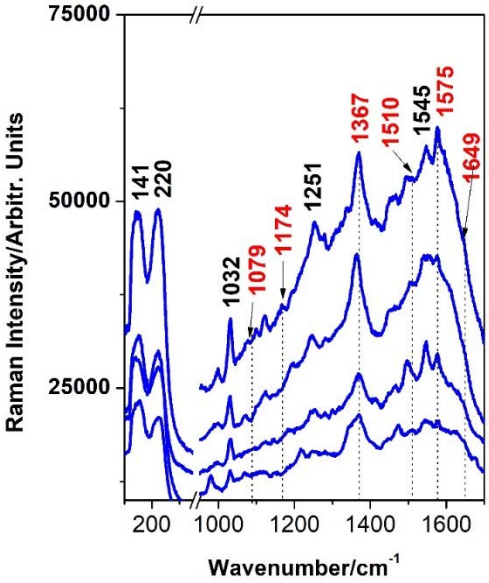


**Fig. 3** SERS spectra of native, uncentrifuged fertilized sea urchin *Paracentrotus lividus* eggs, featuring bands of the fertilization envelope material and low-concentration β-carotene bands (labelled in red).

The signal of centrifuged fertilized eggs featured stronger Raman signal of carotenoids (Fig. 4). Acquisition of pre-resonance Raman signal rather than SERRS presumably occurred due to the phenomenon of SERS damping, where only the first molecular layer adsorbed onto AgNPs gives SERS signal, while increase of carotenoid concentration also increases the Resonance Raman signal, which is stronger and

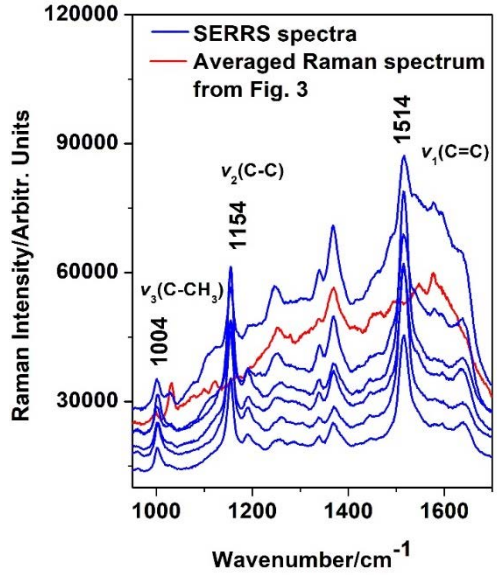


**Fig. 4** Multiple SERRS spectra acquired from sea urchin *Paracentrotus lividus* centrifuged fertilized eggs (blue) compared to a representative spectrum of uncentrifuged fertilized eggs. Note the higher intensity of carotenoid bands at 1004, 1154 and 1514 $cm^{-1}$ after centrifugation (blue spectra).

occludes the comparatively weaker SERS signal [5]. The conclusion of this observation is that the carotenoid concentration increased in or very near to the fertilization envelope.

Carotenoids were previously shown to exhibit remarkable free radical scavenging activity [7], hence their role in chemical defence of cells and tissues. In this study, we have shown, by detecting increased concentration of carotenoids near the fertilization envelope, that carotenoids may also have some role to play in mechanical stress relieving. A previous study that employed centrifugation of sea urchin oocytes has shown that centrifugation temporarily changes the spatial distribution of cell organelles, and that normal organization of the cell interior is restored later [14]. Hence, centrifugation could not have permanently narrowed the perivitelline space and so brought the cell itself closer to the envelope, where AgNPs are aggregated. It is possible that mechanical stress induces chemical insults within the cell, and that is where carotenoids may come into play to remediate oxidative stress.

The localization of SERS signal from the fertilization envelope is supported by the fact that the signal of only low-concentration β-carotene signal along with general indicator bands of proteins [15] was recorded, rather than strong carotenoid signal which would be recorded under Resonance Raman excitation only. The conclusion on increased concentration of carotenoids near the fertilization envelope is supported by the fact that electromagnetic enhancement of atomic vibrations by SPR extends only a few nanometres from the nanoparticles surface, which is much smaller that the width of perivitelline space. Thus, the phenomenon of SERS damping by carotenoids must have occurred near the fertilization envelope.

Detection of increased carotenoid content near the fertilization envelope after the mechanical stress, i.e. centrifugation, raises a new question that has to be investigated by other methods: a physiological mechanism must exist, which rapidly transports carotenoids, which are lipid-soluble, from cells into the envelope, which is not believed to be rich in lipides. Hence the question of means of cellular transport of carotenoids through media where they are not soluble.

## CONCLUSION

In this paper we have shown, using Resonance Raman scattering and Surface-enhanced Raman scattering, that mechanical stress to sea urchin eggs, in form of centrifugation within non-lethal parameters, induces a shift in cellular carotenoids distribution. The carotenoids are engaged presumably because centrifugation induces oxidative stress to the eggs.

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